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Genetically Modified Bacterial Strains and Novel Bacterial Artificial Chromosome Shuttle Vectors for Constructing Environmental Libraries and Detecting Heterologous Natural Products in Multiple Expression Hosts

Asuncion Martinez,* Steven J. Kolvek, Choi Lai Tiong Yip, Joern Hopke, Kara A. Brown,† Ian A. MacNeil,† and Marcia S. Osburne†

Cambridge Genomics Center, Aventis Pharmaceuticals Inc., Cambridge, Massachusetts 02139

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The enormous diversity of uncultured microorganisms in soil and other environments provides a potentially rich source of novel natural products, which is critically important for drug discovery efforts. Our investigators reported previously on the creation and screening of an *Escherichia coli* library containing soil DNA cloned and expressed in a bacterial artificial chromosome (BAC) vector. In that initial study, our group identified novel enzyme activities and a family of antibacterial small molecules encoded by soil DNA cloned and expressed in *E. coli*. To continue our pilot study of the utility and feasibility of this approach to natural product drug discovery, we have expanded our technology to include *Streptomyces lividans* and *Pseudomonas putida* as additional hosts with different expression capabilities, and herein we describe the tools we developed for transferring environmental libraries into all three expression hosts and screening for novel activities. These tools include derivatives of *S. lividans* that contain complete and unmarked deletions of the *act* and *red* endogenous pigment gene clusters, a derivative of *P. putida* that can accept environmental DNA vectors and integrate the heterologous DNA into the chromosome, and new BAC shuttle vectors for transferring large fragments of environmental DNA from *E. coli* to both *S. lividans* and *P. putida* by high-throughput conjugation. Finally, we used these tools to confirm that the three hosts have different expression capabilities for some known gene clusters.

Natural products have been a rich source of pharmaceutical molecules, accounting for greater than 30% of all human therapeutics and more than 60% of antiinfective and anticancer drugs. Despite the advances in high-throughput screening technology and attempts to isolate and culture microorganisms from exotic environments, the discovery of novel natural products remains difficult. However, it has become clear that the vast majority of microorganisms in the environment are still unknown and that most of them are unculturable under standard laboratory conditions (15, 38). Since the number of such "unculturable" microbial species in the soil represents at least 98% of the total population, these species constitute a potentially large untapped pool of novel natural products. To access their genetic information, the DNA of these microorganisms can be isolated directly from environmental samples, cloned into suitable vectors, and expressed in surrogate hosts that can be grown in the laboratory and manipulated genetically (9, 17, 18, 26, 29, 36).

Previously, our investigators and others reported on methods to isolate and clone environmental DNA and screen for novel bioactivities (9, 17, 18, 26, 29) using *Escherichia coli* strains and vectors. Although interesting and novel activities have been expressed and identified in this host, the potential advantage of expanding the range of bacterial hosts to capture additional expression capability is clear. We chose to extend our expression host range to include *Streptomyces lividans* and *Pseudomonas putida*. Actinomycetes have been a major source of natural products, including polyketides and nonribosomal peptides, are capable of supplying a wide variety of precursors and enzymes, and are able to express heterologous polyketides (16). Among the actinomycetes, *S. lividans* is one of the easiest species to manipulate genetically. Our initial work with *Streptomyces* (9) focused on a shuttle cosmid vector which, although useful, required a cumbersome transformation procedure. We therefore sought a more reliable and high-throughput DNA transfer process.

The gram-negative pseudomonads colonize many niches, including soil, fresh water, and biotic and abiotic surfaces (23). They have large genomes (over 6 Mb) and rich metabolic diversity, including gene clusters for degradation of xenobiotics and for production of secondary metabolites such as polyketides and nonribosomal peptides (3, 22, 33, 34). Importantly, many tools have also been developed for pseudomonads (including transformation, conjugation, transposon mutagenesis, and a wide variety of vectors and reporter systems), so that their genetic manipulation is relatively straightforward.

In the present work we describe new tools that facilitate the use of *S. lividans* and *P. putida* as expression hosts for environmental DNA libraries. These tools include derivatives of *S. lividans* that contain complete and unmarked deletions of the *act* and *red* endogenous pigment gene clusters, an improved *P. putida* host strain, and new bacterial artificial chromosome

* Corresponding author. Mailing address: Aventis Pharmaceuticals Inc., Cambridge Genomics Center, 26 Landsdowne St., Cambridge, MA 02139. Phone: (617) 768-4023. Fax: (617) 374-8811. E-mail: Asuncion.martinez@aventis.com.

† Present address: ActivBiotics, Inc., Lexington, MA 02421.

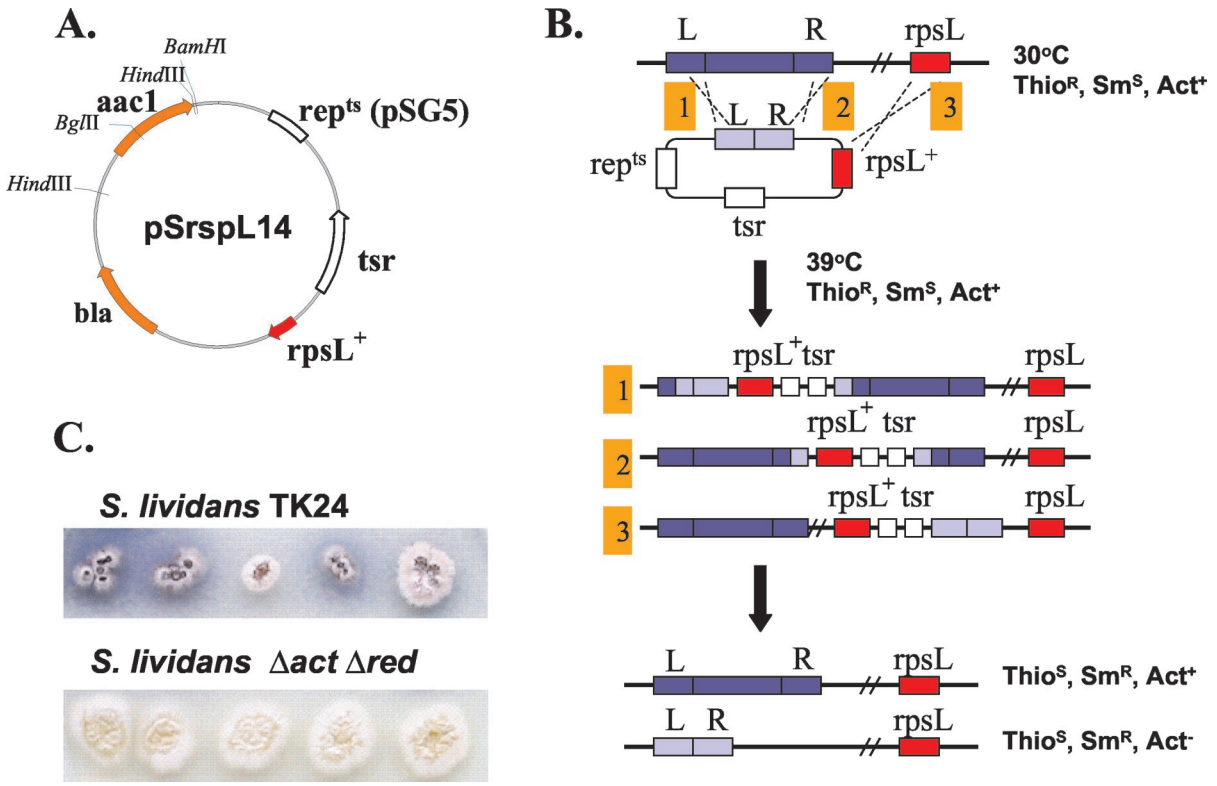

FIG. 1. Deletion of *S. lividans* endogenous antibiotic gene clusters. (A) Gene replacement plasmid pSrpsl14 was constructed by cloning an EcoRI fragment containing the wild-type *rpsL* (Sm^s) gene of *S. coelicolor* A3(2) into pGM160. ts *ori* (pSG5), temperature-sensitive origin of replication in *Streptomyces*; *bla*, Amp^r (*E. coli*); *tsr*, $Thio^r$ (*Streptomyces*); *aac1*, gentamicin resistant (*E. coli* and *Streptomyces*). Restriction sites available for cloning are indicated. (B) Schematic diagram of the method used to delete the *act* cluster from *S. lividans* TK24 using pΔact18. See text for details. (C) Pigment production by *S. lividans* TK24 (top) and *S. lividans* ΔactΔred (bottom) grown in R5 plates for 7 days and photographed.

(BAC) shuttle vectors for transferring large fragments of environmental DNA from *E. coli* to *S. lividans* and *P. putida* by conjugation. Finally, we report on a high-throughput method for transferring environmental DNA libraries into both *S. lividans* and *P. putida*, using the same shuttle BAC vector library, and demonstrate the utility of screening for expression of heterologous compounds in all three expression hosts.

MATERIALS AND METHODS

Bacterial strains and plasmids. The strains and plasmids used in this study are listed in Table 1.

Growth conditions. *E. coli* DH10B was grown in Luria-Bertani (LB) medium (27) supplemented with antibiotics as indicated. *Streptomyces coelicolor* and *S. lividans* strains were grown at 29°C in YEME, GYM, R2, or R5 medium as reported previously (16). For conjugations, we used a modified R2 medium in which sucrose was omitted. *P. putida* was routinely grown in LB supplemented with Fe-citrate (6 mg/liter) at 30°C, except for selection of exconjugants, where we used M9 benzoate medium (10). For antibacterial and antifungal screens, *Bacillus subtilis* and *Candida albicans* were grown in LB with chloramphenicol (Cam; 15 μg/ml) and yeast-peptone-dextrose (YPD; 10 g of yeast extract, 20 g of peptone, 20 g of dextrose per liter), respectively. Antibiotics were used as indicated (concentrations are given in micrograms per milliliter).

Plasmid constructions. Standard methods were used for DNA isolation and recombinant procedures (16, 27). PCR was performed using *Vent* polymerase (New England BioLabs) according to the manufacturer's instructions, with the addition of 7.5 to 10% dimethyl sulfoxide. Strategies for constructing plasmids were as follows.

(i) pSrpsL6 and pSrpsL14. The wild-type *rpsL* gene was amplified by PCR from *S. coelicolor* A3(2) using primers rpsL5′ (5′GGAATTCCTTCGTCCGCC ACGACACG3′) and rpsL3′ (5′GGAATTCCGTCTTGCCCGCGTCGATG3′). The 1.3-kb *rpsL* fragment was digested with EcoRI (restriction sites underlined) and cloned into the EcoRI site of pBKII SK^- (Stratagene), yielding pBKrpsL122. The *rpsL* fragment of pBKrpsL122 was isolated by EcoRI digestion and cloned into the EcoRI site of pGM160 (21), resulting in plasmids pSrpsL14 and pSrpsL6, with the insert in opposite orientations (Fig. 1A).

(ii) pΔact18. The *actVIBA* genes (left end of the *act* cluster [11]) were amplified by PCR from *S. lividans* TK24 using the primers actVI5′ (5′GAAGATCT TCGGCAGCGCGTCAGGGTGTCA3′) and actVI3′ (5′GGAATTCCTACTG CCTGGTGCTCACCGTCCAC3′) and digested with BglII and EcoRI. The *actVB* ORF11 and ORF12 genes at the right end of the *act* cluster (19) were also PCR amplified from TK24 using primers act.lysR25′ (5′GGAATTCCACGAG GGTGGTTGGCGTCGGAACAAGGC3′) and act.lysR23′ (5′CGGGATCCCA GGAAGCACAGGACGCCGAGGACGAAC3′) and digested with EcoRI and BamHI. The *actVI* and *actVB* fragments were ligated with pGM160rpsL14 digested with BamHI and BglII and dephosphorylated with shrimp alkaline phosphatase. The resulting plasmid, pΔact18, was used to delete the *act* cluster from *S. lividans*.

(iii) pΔred. The *redD* gene was PCR amplified from TK24 using the primers redD5′.Xover (5′GACGGCCAAGCTTCCTCGACCTTGTGGACCTCGTCG GTGCGCATCA3′) and redD3′.Xover (5′GATCATCGGGTCGTCTGTTTAA ACGGTCGTCAGGCGCTGAGCAGGCTGGTGT3′). The PCR product was cloned into pCR4-Blunt-TOPO (Invitrogen), yielding pTOPO-TK3. The *S. lividans* homolog of SC10A5.02 (chosen as the right end of the *red* cluster) was amplified using the primers red.oxidase.5′.Xover (5′CGCCTGACGACCGTTT AAACAGACGACCCGATGATCCCCAACCAGTGG3′) and red.oxidase.3′ (5′CGGGATCCCGCGGGGTCAGTACACGTAGGGGACGAACTTC3′) and cloned into the EcoRV site of pBK-SK^- (Stratagene) to give pBK-TK3. pΔred was created by ligating the *redD* fragment (as a 1.9-kb HindIII-PmeI fragment of pTOPO-TK3) and the SC10A5.02 homolog fragment (a 1.9-kb PmeI-BamHI

TABLE 1. Bacterial strains and plasmids

Plasmid or strain	Description	Source or reference
Plasmids		
pBKII SK$^-$	General cloning vector; *bla* (Amp^r)	Stratagene
pCR4Blunt-TOPO	PCR product cloning vector	Invitrogen
pDNR-1	cre-*lox* donor vector; *bla* (Amp^r), *sacB* (sucrose^s)	Clontech
pBKrpsL	1.3-kb fragment containing *S. coelicolor rpsL* cloned into EcoRI site of pBKII SK$^-$	This work
pGM160	Ts *Streptomyces* pSG5 ori; *aac1 bla* (Amp^r) *tsr* (Thio^r)	21
pSrpsL6/14	1.3-kb EcoRI fragment of pBKrpsL cloned into pGM160; *bla* (Amp^r) *tsr* (Thio^r) *rpsL* (Sm^r)	This work
pΔact18	pSrpsL14 derivative containing *actVB* and *actVI* fragments of *S. lividans act* cluster; *bla* (Amp^r) *tsr* (Thio^r) *rpsL*$^+$ (Sm^s)	This work
pTOPO-TK3	*redD* PCR product cloned in pCR4Blunt-TOPO	This work
pBK-TK4	SC10A5.02 PCR product cloned in pBKII SK$^-$	This work
pΔred	pSrpsL6 derivative containing the *redD* fragment from pTOPO-TK3 and the SC10A5.02 fragment of pBK-TK4	This work
pOJ446-22-16	Contains granaticin gene cluster from *S. violaceoruber* Tu22	2
pOJ436	*Streptomyces* conjugative cloning vector; *aac(3)IV* (Apra^r)	5
pBeloBac11	BAC cloning vector; Cam^r; single copy	31
pMBD7	6.6-kb SpeI-DraI fragment of pOJ436 cloned in pDNR1; Apra^r Amp^r	This work
pMBD9	pMBD7 derivative with BstXI site removed; Apra^r Amp^r	This work
pMBD12	pMBD9 derivative with BamHI site removed; Apra^r Amp^r	This work
pMBD10	pMBD7 *Streptomyces* cassette in pBeloBac11; Apra^r Cam^r; single copy	This work
pMBD13	pMBD9 *Streptomyces* cassette in pBTP3; Apra^r Cam^r; high copy	This work
pMBD14	pMBD12 *Streptomyces* cassette in pBeloBac11; Apra^r Cam^r; single copy	This work
pBTP3	Modified BAC vector; Cam^r; high copy, one *lox*P site	I. MacNeil (Aventis)
MG1.1	Antibacterial soil BAC clone	17
pSMG1.1	pMBD9 CIS cassette in MG1.1; Cam^r Apra^r	This work
pGran	38-kb granaticin cluster from pOJ446-22-16 cloned into pBTP3	This work
pSGran	pMBD9 CIS cassette in pGran; Apra^r Cam^r	This work
p1000	Source of ϕCTX (*P. aeruginosa* phage) *attP* site	37
pIHB	pUC plasmid encoding the ϕCTX integrase	37
pCR-BluntII Topo	PCR cloning vector	Invitrogen
pMON5122	Source of DAPG cluster	35
pSDAPG	DAPG cluster cloned into pMBD13; Apra^r Cam^r	This work
pTOPOattB	ϕC31 *attB* of *S. lividans* cloned into pCR-BluntII Topo; Kan^r	This work
p2.10	ϕCTX *attP* site cloned into pTOPOattB; Kan^r	This work
Strains		
E. coli ET12567/pUB307	Donor strain for conjugative transfer into *Streptomyces;* Cam^r Kan^r; lacking *dam* and *dcm*	12
E. coli DH10B	General cloning strain, dam$^+$, dcm$^+$, restriction-	Gibco/BRL
E. coli DH10B/pUB307	Donor strain for conjugative transfer; Cam^r Kan^r Dam$^+$ Dcm$^+$	This work
S. coelicolor A3(2)	*rpsL*$^+$	John Innes Centre
S. lividans TK24	*rpsL* (Sm^r) Act$^+$ Red$^+$	John Innes Centre
S. lividans Δ*act*	TK24 derivative with a deletion of the *act* cluster; Red$^+$	This work
S. lividans Δ*red*	TK24 derivative with a deletion of the *red* cluster; Act$^+$	This work
S. lividans Δ*act*Δ*red*	TK24 derivative with deletions of the *act* and *red* clusters	This work
P. putida KT-2440	Laboratory strain of *P. putida*	13
P. putida MBD1	KT-2440 derivative; Kan^r; ϕC31 *attB* site$^+$	This work
P. fluorescens ATCC49323	Source of DAPG cluster	ATCC
B. subtilis BR151/pPL608	Sensitive strain used for antibiotic bioassays	Bacillus Genetic Stock Center
C. albicans NCCLS11	Used for antifungal bioassays	ATTC

fragment from pBK-TK4) into pGM160rpsL6 that had been digested with HindIII and BamHI. pΔred was used to delete the *red* cluster from *S. lividans*.

(iv) pMBD7, -9, and -12. pMBD7 was constructed by cloning a 6.6-kb SpeI-DraI fragment of pOJ436 (5) into a 4.1-kb XbaI-partial PvuII fragment of pDNR-1 (Clontech). DH10B transformants were selected on agar plates containing LB with ampicillin (Amp; 100 μg/ml) and apramycin (Apra; 50 μg/ml) and tested for sensitivity to 7% sucrose (conferred by the *sacB* gene) prior to restriction analysis. pMBD9 is a derivative of pMBD7 in which a BstXI site at the end of the *aac(3)IV* gene has been removed by digestion with BstXI, blunting of the ends with T4 DNA polymerase, and religation. pMBD12 is a derivative of pMBD9 in which the unique BamHI site has been removed by the method described above for pMBD9.

(v) pGran. A 38.2-kb EcoRV fragment of pOJ446-22-24, containing the granaticin gene cluster for *Streptomyces violaceoruber* Tu22 (2), was cloned into pBTP3 (U.S. patent application 09/596,114) by the adaptor cloning method. Briefly, pOJ446-22-24 was digested with EcoRV, ethanol precipitated, and ligated to BstX1 adaptors (N418-18; Invitrogen) in 1× blunt-end ligation buffer (50 mM Tris-HCl [pH 7.8], 50 μM ATP, 10 mM β-mercaptoethanol, 5 mM $MgCl_2$), 15% (wt/vol) polyethylene glycol 8000, 400 U of ligase at 16°C overnight. The granaticin fragment was purified from a pulsed-field gel (electrophoresis in a 1% low-melting-point agarose [0.5× Tris-borate-EDTA], 0.1 to 35 s switch time; 6 V; 14°C for 12 h). The gel slice was dialyzed against Tris-EDTA buffer for 2 h prior to digestion with Gelase (Epicenter), according to the manufacturer's recommendations, and ligated to 20 ng of BstX1-cut pBTP3-43 vector (10:1 vector/insert molar ratio) at 16°C for 6 h. After electroporation into ElectroMax DH10B cells, transformants were selected on LB agar plates containing Cam (12.5 μg/ml).

(vi) pSDAPG. A 6.5-kb XbaI-EcoRI fragment containing the locus encoding 2,4-diacetylphloroglucinol synthesis (DAPG cluster) was excised from pMON5122, blunted using T4 DNA polymerase, and ligated to BstX1 adaptors (N418-18; Invitrogen) in 1× blunt-end ligation buffer (50 mM Tris-HCl [pH 7.8], 50 μM ATP, 10 mM β-mercaptoethanol, 5 mM $MgCl_2$), 15% (wt/vol) polyethylene glycol 8000, 400 U of ligase at 16°C overnight. The DAPG fragment was gel purified and ligated to 20 ng of BstX1-cut pMBD13 vector (10:1 vector/insert molar ratio) at 16°C for 6 h. After electroporation into ElectroMax DH10B, transformants were selected on LB Cam (12.5 μg/ml) agar plates.

Transfer of the *Streptomyces* cassette to BAC vectors. The *Streptomyces* cassette in plasmids pMBD7, pMBD9, and pMBD12 was transferred to BAC vectors by in vitro cre-*lox* recombination using the Creator pDNR-1 cloning kit (Clontech) according to the manufacturer's instructions. Recombination products were selected after transformation into ElectroMax DH10B cells (Gibco/BRL) by plating on LB agar containing 7% sucrose, Cam (12.5 μg/ml), Apra (30 μg/ml). Transfer of the pMBD7 and pMBD12 cassettes into pBeloBac11 yielded pMBD10 and pMBD14, respectively. Transfer of the pMBD9 cassette to pBTP3, MG1.1, and pGran yielded pMBD13, pSMG1.1, and pSGran, respectively.

Strain constructions. (i) *S. lividans* ΔactΔred. Plasmids pΔact and pΔred were used separately to transform TK24 (streptomycin resistant [Sm^r]) protoplasts by standard methods (16). Transformants were selected in R2YE containing 50 μg of thiostrepton (Thio)/ml at 29°C. Individual transformants were grown twice in YEME containing Thio (8 μg/ml) for 3 to 5 days at 29°C, homogenized, and plated in R2YE plus Thio (50 μg/ml) at 39°C to select for single crossover events. Four to six clones selected at 39°C were grown for 5 days at 39°C in YEME plus Thio (8 μg/ml). A 100-μl aliquot of the culture was inoculated into 100 ml of YEME without Thio. Second crossover events resulting in excision of plasmid sequences were selected by plating on GYM (30) plus Sm (50 μg/ml) at 39°C. Each clone was then tested for Thio sensitivity and pigmented antibiotic production on R2 plates. The presence or absence of each antibiotic cluster on the chromosome was verified by PCR analysis using the following primers: Δact.1 (GTGGGTACCCGTGGGTACCTGTGCTGCTTT), Δact.2 (TTGTTGACCA GTACGTCCACCCTGCCGTGC), Δact.3 (AGATGCAGAAGCTGGACGGC CGTGACTTCG), Δred.1 (GGCCCTGGAGGATCTCATCAGCGCGATG TT), Δred.2 (TAGAGGGCGGACATCCCGACGATGGCGAT), and Δred.3 (AGCCGTGGTACGGGCATTCGATGGTGTTGC).

(ii) *P. putida* MBD1. The ϕC31 *attB* sequence of *S. lividans* was PCR amplified using the primers attB5′ (ACCATCGTGATCGGCGTGTGCGTGATGCCG) and attB3′ (GCCCGTGATCCCGATGTTCACCGGCCTGAAG) and *Vent* polymerase (New England BioLabs). The resulting 939-bp fragment was cloned into pCR-BluntII Topo (Invitrogen), yielding pTOPOattB. Next, a 1.1-kb PstI fragment from plasmid p1000 containing the ϕCTX (*P. aeruginosa* phage) *attP* site (37) was cloned into the PstI site of pTOPOattB. The resulting plasmid, p2.10, was cotransformed with pIHB (37) into electrocompetent *P. putida* KT-2440. After electroporation, cells were recovered in 2 ml of SOC (27) at 30°C prior to selection. Transformants in which p2.10 had integrated at the ϕCTX *attB* site were selected on LB containing 25 μg of kanamycin (Kan)/ml, resulting in strain *P. putida* MBD1. The presence of the ϕC31 *attB* site in the *P. putida* chromosome was verified by Southern hybridization using the 938-bp ϕC31 *attB* fragment as a probe.

Southern hybridizations. Chromosomal DNA was prepared using DNeasy columns (Qiagen). Southern hybridization was performed by standard procedures (27). BamBAC8 plasmid DNA and the gel-purified ϕC31*attB* fragment from pTOPOattB were used as probes. Probes were labeled with [α-^{32}P]dCTP using the Readyprime II kit (Amersham).

Environmental DNA library. Megabase environmental DNA was isolated from a local soil sample as previously described (17). The megabase DNA plug was dialyzed against 15 ml of 1× BamHI buffer with bovine serum albumin at 4°C for 1 h. The gel slice was melted at 65°C for 5 min, equilibrated to 37°C for 5 min, and digested with 0.8 U of BamHI at 37°C for 1 h. The digestion was stopped by addition of EDTA (50 mM final concentration). After pulsed-field electrophoresis in 1% low-melting-point agarose (0.5× Tris-borate-EDTA; 0.5 to 35 s switch time; 6 V; 14°C for 10 h), a gel slice containing 50 to 100 kb of DNA was excised from the gel and digested with Gelase (Epicenter). Two nanograms of soil DNA was ligated at 16°C overnight with 20 ng of pMBD14 digested with BamHI and dephosphorylated with calf intestinal phosphatase. Two microliters of the ligation mixture was used to transform ElectroMax DH10B cells (Gibco/BRL) by electroporation (0.2-cm cuvette; 2.5 kV). Transformants were selected on LB Cam (12 μg/ml) Amp (30 μg/ml) agar plates.

Standard conjugations into *S. lividans* and *P. putida*. For *S. lividans*, conjugations of individual plasmids were performed as described previously (16) using ET12567/pUB307 or DH10B/pUB307 as the donor strain. For *P. putida* standard conjugations, the *E. coli* donor strain DH10B/pUB307 containing the BAC construct to be transferred was grown overnight at 37°C in LB containing Cam (12 μg/ml), Apra (30 μg/ml), and Kan (50 μg/ml). The recipient, *P. putida* MBD1, was also grown overnight at 30°C in LB Kan (50 μg/ml). Donor and recipient were diluted 1:100 into fresh medium and grown for 4 h. The recipient was incubated at 42°C for 15 min to inactivate restriction enzymes. Donor and recipient (1:3) were mixed in a microcentrifuge tube, centrifuged for 1 min, and resuspended in 50 μl of LB. The mix was placed on an LB agar plate and incubated at 30°C for 24 h, and cells were then scraped from the plate and resuspended in 1 ml of LB. Dilutions were plated on LB agar plus nalidixic acid (Nal; 20 μg/ml) and Apra (25 μg/ml). Exconjugants were picked after 2 days at 30°C.

High-throughput transfer of BAC libraries into *S. lividans* Δ*act*Δ*red* and *P. putida* MBD1. High-throughput transfer of environmental libraries was performed as follows. Pools of DH10B environmental library clones were grown in selective medium as above, and plasmid DNA was isolated. The pooled plasmids were then used to transform electrocompetent DH10B/pUB307. Transformants were picked with a Q-bot robot (Genetix) into 96-well deep plates containing LB CHL 12, APRA 30, KAN 50, and grown overnight at 37°C to a final optical density at 600 nm of 3.5 to 4.0. Donor *E. coli* cells were then diluted 1:10 using LB without antibiotics in a 96-well plate. For conjugations into *S. lividans*, a 96-pin stamper (no. 140500; Boekel Scientific) was dipped into the donor wells and then into the recipient wells containing 100 μl of heat-shocked *S. lividans* Δ*act*Δ*red* recipient spores (10^8/ml). The same stamper was used to deliver droplets of the donor-recipient mix onto R2 plates (minus sucrose). The droplets were allowed to dry before overnight incubation at 30°C. Q-bot plates were then dried for 2 h under a tissue culture hood. Fifteen milliliters of an aqueous solution containing selective agents (75 μg of Apra, 25 μg of Nal, and 50 μg of hygromycin/ml, final concentrations) was added by flooding the plate and rotating it continuously until the liquid was absorbed. Plates were then incubated further for 3 to 4 days. Soil DNA exconjugants were replicated once onto R5 containing Nal (25 μg/ml) and Apra (75 μg/ml) before replicating onto the screening medium.

For *P. putida* high-throughput conjugations, cultures of the *E. coli* donor strain DH10B/pUB307 containing library clones were prepared and diluted 1:10 as described above for the *Streptomyces* conjugations. A 96-pin replicator was used to deliver an aliquot of the donor cultures into 96-well plates containing 50 μl of a *P. putida* MBD1 exponential culture that had been incubated at 42°C for 15 min to inactivate restriction systems. The same replicator was used to deliver aliquots of the mixes onto an LB Q-bot plate, which was then incubated overnight at 30°C. *P. putida* exconjugants containing library clones were selected by replicating the colonies onto an M9 benzoate plate (10) with Apra (25 μg/ml) and Nal (20 μg/ml), on which only exconjugants can grow. Exconjugant colonies were visible after 2 to 3 days of incubation.

High-throughput antibacterial and antifungal screens. *S. lividans* Δ*act*Δ*red* exconjugants were grown on R5 plates (16) for 7 days at 30°C. Plates were then overlaid with top agar (27) containing exponentially growing *B. subtilis* strain

BR151/pPL608 (Bacillus Genetic Stock Center, Columbus, Ohio) or *C. albicans* NCCLS11 (ATCC 90028) and incubated overnight at 30°C followed by several days at room temperature. Clones producing antibacterial or antifungal activities were identified by a zone of inhibition in the lawn surrounding the clone. For *P. putida* MBD1, exconjugants were picked from the selection plate after conjugation onto a fresh M9 benzoate plate containing Nal (20 μg/ml) and Apra (25 μg/ml), using a 96-pin replicator. This second round of growth in the presence of selection was used to eliminate residual donor *E. coli* cells. A 96-pin replicator was used again to inoculate shallow 96-well plates containing 150 μl of liquid LB medium supplemented with Fe-citrate (6 mg/liter). Cultures were grown at 29°C in a humidified container for 5 to 7 days and then dried using a Savant Speed-Vac Plus SC210A. One volume of methanol was added to the pellets. After 15 min at room temperature in covered plates, the extracts were removed with a multichannel pipettor, avoiding the solid residue. Extracts were divided in two and dried to completion in the Speed-Vac prior to assays. For antibacterial assays, extracts were resuspended in 145 μl of LB. Five microliters of a 1:10 dilution of an early-log-phase culture of *B. subtilis* BR151/pPL608 was added to the resuspended extracts, and the plates were incubated at 37°C overnight with shaking (250 rpm). Growth of the *B. subtilis* tester strain was evaluated visually. For antifungal assays, extracts were resuspended in 145 μl of YPD medium (10 g of yeast extract, 20 g of peptone, 20 g of glucose) plus 5 μl of *C. albicans* ATCC 90028 from a frozen glycerol stock, diluted 1:100. Plates were incubated at 35°C overnight. Growth of the *C. albicans* tester strain was evaluated visually.

Preparation of extracts and HPLC analysis. Inertsil ODS-3 (5 μm; 120 Å, 150 by 4.6 mm; GL Sciences) and Polaris C-18A (5 μm; 120 Å; 4.6 by 150 mm; Metachem) columns were used for analytical reverse-phase high-pressure liquid chromatography (HPLC) on a Waters 600 system with 996 PDA detector (210 to 610 nm; 1.2-nm resolution; Millennium 4.0 software). The mobile phase was 0.08% trifluoroacetic acid (TFA) in water (solution A) and 0.08% TFA in acetonitrile (solution B).

To analyze antibiotic production in *S. lividans*, the appropriate strains were grown in 25 ml of YEME with Apra (50 μg/ml) at 30°C for 4 days. Cultures were lyophilized (Labconco Freezone 4.5) and extracted with methanol-ethyl acetate (3:1). Extracts were filtered, concentrated (N_2 stream; Pierce Reacti-Therm), cleaned by solid-phase extraction (Waters SepPak C_{18} cartridges; 3 ml; 200 mg), and dried under an N_2 stream. Extracts were redissolved in 1 ml of methanol and filtered (Whatman 4-mm, 0.2-μm polytetrafluoroethylene syringe filters) prior to HPLC analysis. Elution started with an solution A/solution B ratio (A/B) of 95:5 for 2 min, and then a linear gradient was run from an A/B of 95:5 to an A/B of 2:98 for 25 min with an 8-min hold at an A/B of 2:98. The flow rate was 1.5 ml/min, and the injection volume was 25 μl. The absorbance of the effluent at 240 and 500 nm was recorded.

For HPLC analysis of secondary metabolite production in *P. putida* MBD1, liquid cultures of *P. putida* MBD1 exconjugants containing pMBD14, pSgran, pSMG1.1, or pSDAPG were grown for 7 days at 27°C in 50 ml of YM medium (1) containing Apra (25 μg/ml). Ethyl acetate extracts were prepared as described previously (6). The extracts were reconstituted in 1 ml of H_2O-CH_3CN (50:50 [vol/vol]) containing 0.08% TFA. Samples were filtered (Whatman 4-mm, 0.2-μm polytetrafluoroethylene syringe filters) prior to HPLC analysis. Elution began with 100% solution A for 2 min, and then a linear gradient was run from 0 to 100% solution B for 20 min with a 10 min hold at 100% B. The flow rate was 1 ml/min, and the injection volume was 10 μl. Identification of 2,4-diacetylphloroglucinol (DAPG) was based on the UV spectrum (6).

RESULTS

Deletion of *S. lividans* endogenous antibiotic gene clusters. Optimally, host strains used to express environmental libraries should lack endogenous activities and pigments that may both interfere with the detection of heterologous compounds and potentially waste available metabolites that could be used to produce heterologous compounds. To obtain such appropriate strains of *S. lividans*, the *act* and *red* gene clusters encoding actinorhodin and undecylprodiginine, respectively, were deleted from the *S. lividans* chromosome by positive selection of unmarked allelic exchange mutants. This method involves a two-step strategy that combines the use of a temperature-sensitive replicon and a counterselectable marker (reviewed in reference 25). It has been shown previously that the wild-type *rpsL* gene of *Streptomyces roseosporus* is counterselectable in an Sm^r background since, as in other bacteria, it confers dominant sensitivity to Sm (14). Our gene replacement vector, pSrpsL14 (Fig. 1A), contains the wild-type *rpsL* gene of *S. coelicolor* A3(2) cloned into pGM160, a shuttle vector with an SG5 *ori* which is naturally temperature sensitive for replication in *S. lividans* (21). The scheme for selection of unmarked mutations was analogous to that described for *Mycobacterium tuberculosis* (24). The ends of the well-defined *act* cluster, the *actVIAB* genes (left end of the cluster [11]), and the *actVB* ORF11-12 genes (right end of the cluster [19]) were cloned into gene replacement vector pSrpsL14 (Fig. 1A) to yield vector pΔact18, which was then used to effect the deletion of the *act* cluster as shown schematically in Fig. 1B. Briefly, pΔact18 was introduced into TK24 by transformation, and transformants were selected by resistance to Thio at 29°C, the permissive temperature for plasmid replication. Single crossover events resulting in integration of the plasmid into the chromosome were selected with Thio at 39°C. The three possible integration products are shown in Fig. 1B. After a round of growth in liquid medium at 39°C without antibiotic selection, those cells that had undergone a second crossover event leading to the excision and loss of the plasmid-borne *rpsL* gene were selected by plating on Sm medium at 39°C. Using this method, 3 out of 12 Sm-resistant clones contained an unmarked deletion of the *act* cluster, as determined by PCR screening. Using the complete genome sequence of *S. coelicolor* A3(2) as reference (4), the resulting Δ*act* deletion encompasses 24.2 kb (nucleotides 143959 to 168217; GenBank accession number SCO939122).

Although the *red* cluster is not as well characterized as the *act* cluster, it is known that the *S. coelicolor red* genes are clustered in a region of approximately 37 kb, with the pathway-specific regulator *redD* at one end (8, 18). We used data from the *S. coelicolor* sequencing project (http://www.sanger.ac.uk/Projects/S_coelicolor/) to define the right end of the desired *red* cluster deletion. We chose SC10A5.02, which encodes a probable oxidase (the last clearly recognizable putative enzyme), as the right end of the *red* cluster deletion. According to a recent analysis by Cerdeño et al. (7), the right end of the *red* cluster extends past SC10A5.02 (*redG*) to include the next gene, SC10A5.02 (*redF*), which they proposed to be an oxidoreductase. Here, the *S. lividans redD* and SC10A5.02 (*redG*) homologs defined the cluster ends in the gene replacement vector pΔred. This plasmid was used to delete the *red* cluster from *S. lividans* TK24 and Δ*act* by the method described for the *act* cluster, yielding *S. lividans* Δ*red* and Δ*act*Δ*red*, respectively. The presence of the *red* cluster deletion in the new strains was verified by PCR. Using the complete genome sequence of *S. coelicolor* A3(2) as reference (4), the resulting Δ*red* deletion encompasses 28.6 kb (nucleotides 144623 to 173286; GenBank accession number SCO939125).

Figure 1C shows the antibiotic production phenotypes of *S. lividans* Δ*act*Δ*red* and TK24. As expected, Δ*act*Δ*red* did not produce actinorhodin or undecylprodiginine and thus provides an improved background for heterologous natural product expression and analysis. Both the double and single cluster deletion strains grew and sporulated as well as the TK24 parent strain (data not shown).

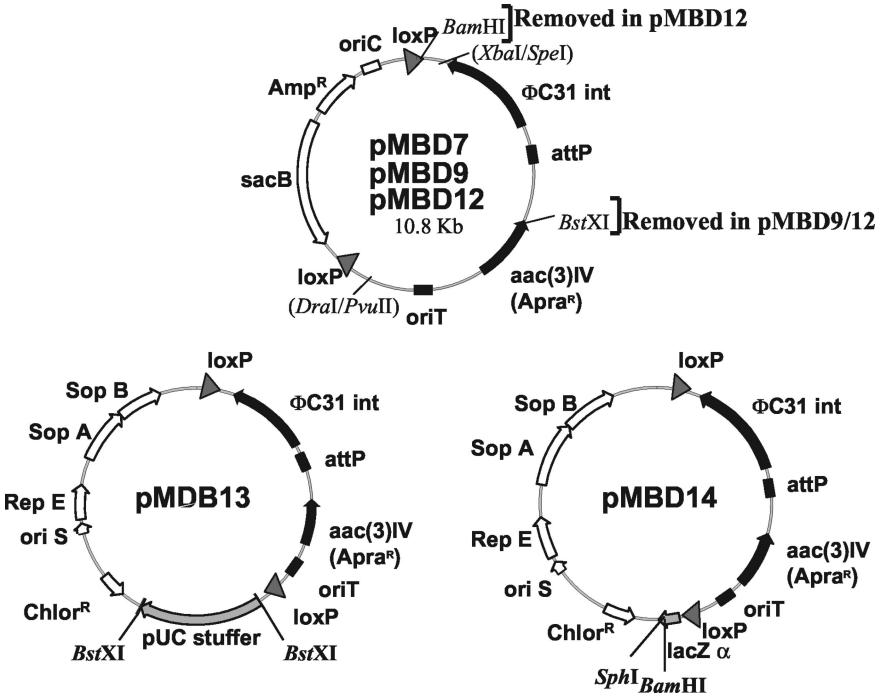

FIG. 2. Plasmids containing CIS cassettes. pMBD7, -9, and -12 (top) are CIS cassette donor plasmids; CIS cassettes contain ϕC31 integrase (*int*) and attachment site (*attP*), *aac3(IV)* for APRA^r, flanked by *loxP* sites; *sacB* confers sensitivity to sucrose. pMBD13 (bottom left) is a BAC plasmid containing the pMBD9 CIS cassette; BstXI sites can be used for cloning by the adaptor method (see Materials and Methods). pMBD14 (bottom right) is a pBeloBac11 derivative with the CIS cassette from pMBD12; unique SphI and BamHI sites within *lacZ*α are available for cloning.

***E. coli-S. lividans* conjugative BAC vectors.** We constructed a new series of plasmids (pMBD7, -9, and -12) (Fig. 2) that encompass the elements required for conjugation of DNA into *Streptomyces* (*oriT*) and subsequent DNA integration into the chromosome (the ϕC31 integrase and attachment site and an Apra resistance marker), all flanked by *loxP* sites. These plasmids can act as donors in an in vitro cre-*lox* recombination reaction (28) to transfer the sequences between the two *loxP* sites (the CIS cassette [conjugative and integrative into *Streptomyces*]) to any *loxP*-containing plasmid. Plasmids pMBD7, pMBD9, and pMBD12 were conjugated into *S. lividans* using *E. coli* ET12567/pUB307 (12) as a donor strain. Conjugation efficiency was similar to that of the parent plasmid, pOJ436 (10^{-5} to 10^{-6} exconjugants per recipient under our conditions), indicating that the CIS cassettes in the donor plasmids were fully functional.

We then transferred the CIS cassette to several BAC constructs by cre-*lox* recombination and measured their efficiency of conjugation into *S. lividans*. Vector pMBD10, derived from *E. coli* plasmid pBeloBac11 (31), was conjugated into *Streptomyces* with high efficiency (10^{-4} to 10^{-5}). Similar high efficiency of conjugal transfer was measured for BAC plasmids pSMG1.1 (containing a 27-kb soil DNA fragment encoding antibacterial activities in *E. coli* [17]) and pSGran (which contains a 38-kb fragment encoding the granaticin gene cluster of *S. violaceoruber* Tu22 [2]). These results demonstrated that the CIS cassette confers all the functions required for efficient mobilization of single-copy BAC vectors with inserts of at least 38 kb.

Two additional BAC vectors, pMBD13 and pMBD14, were built in order to facilitate the construction of DNA libraries (Fig. 2). pMBD13 is designed for cloning using BstXI adaptors, while pMBD14 contains unique BamHI and SphI sites within the *lacZ* α-complementation region, permitting blue-white color selection of recombinant clones. The BamHI site in pMBD14 was used to construct a 13,000-clone soil DNA library (the BamBAC library), with insert sizes ranging from 11.5 to at least 85 kb (see Materials and Methods). Individual clones from this library were used to test the size limit for conjugation into *S. lividans*, comparing two different *E. coli* donor strains (ET12567/pUB307 [Dam$^-$ Dcm$^-$], which is used routinely to transfer DNA into methyl DNA-restricting streptomycetes [12], and DH10B/pUB307). Although DH10B is not DNA methylation deficient, we reasoned that it could be a more suitable donor for the transfer of libraries into *S. lividans*, which is largely nonrestricting, since it is known to be particularly efficient for the uptake of large DNA (40) and thus might discriminate less against large clones in the BamBAC library. Both donor strains efficiently transferred BamBAC clones with inserts as large as 85 kb, significantly larger than any reported previously. Southern analysis of several exconjugants showed that the 85-kb insert construct integrated into the *S. lividans* chromosome, although some contained deletions at one integration junction (data not shown). However, most of the environmental insert DNA was present in the chromosome of the exconjugants, resulting in long contiguous stretches of environmental DNA integrated in the new host and available for expression. Similar analysis showed that a BamBAC construct containing a 38-kb insert (BamBAC8) could be success-

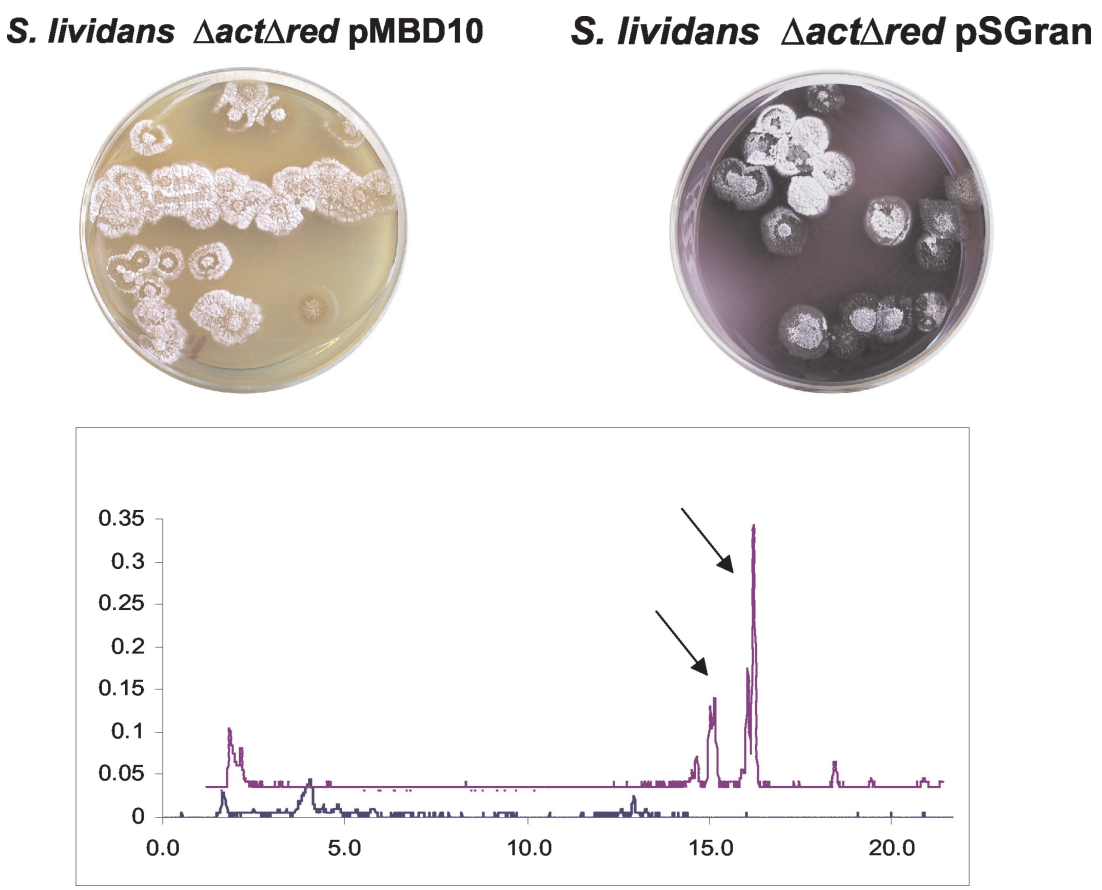

FIG. 3. Heterologous granaticin production from pSgran in *S. lividans* Δ*act*Δ*red*. *S. lividans* Δ*act*Δ*red* exconjugants containing the negative control construct pMBD10 (left) or pSgran (right) were analyzed for granaticin production. Purple pigment production in R5 plates is shown on the top. HPLC profiles of extracts of liquid (YEME) cultures are shown on the bottom. The arrow indicates granaticin peaks in the extract of the pSGran exconjugant (top trace), which were absent in the control (bottom trace).

fully transferred and stably maintained in its entirety in *S. lividans* Δ*act*Δ*red* (see Fig. 5).

Heterologous antibiotic expression in *S. lividans* Δ*act*Δ*red*. The conjugative BAC construct pSGran contains the *S. violaceoruber* Tu22 granaticin cluster (2). Conjugation of pSGran into the unpigmented *S. lividans* Δ*act*Δ*red* strain led to production of purple pigment that was clearly detectable both visually and by HPLC analysis (Fig. 3). One hundred percent of the *S. lividans* exconjugants tested produced the pigment, indicating that the new vectors were stable in both *E. coli* and *S. lividans*. The absence of endogenous antibiotics in *S. lividans* Δ*act*Δ*red* also allowed the clear detection of the antibiotic activity of granaticin against *B. subtilis* (data not shown). These results confirmed that biosynthetic clusters can be efficiently introduced into the unpigmented *S. lividans* Δ*act*Δ*red* strain by conjugation and detectably expressed from the chromosome.

Construction of a *P. putida* host strain for expression of environmental libraries. To further extend our host range for expression of environmental libraries, we chose the well-characterized nonpathogenic soil organism *P. putida* KT-2440 (22, 34). We then engineered the *P. putida* strain to accept our *E. coli-Streptomyces* BAC shuttle vectors and libraries by inserting the ϕC31 (*Streptomyces* phage) *attB* site into the chromosome of *P. putida*, in the hope that the ϕC31 integrase and *attP* site could be used by this host. We used the site-specific integration system of *P. aeruginosa* phage ϕCTX (37) to deliver the ϕC31 *attB* site to the *P. putida* chromosome, according to the scheme shown in Fig. 4. The resulting strain was named *P. putida* MBD1.

Crude methanol extracts of *P. putida* MBD1 were tested for antibacterial and antifungal activity (see Materials and Methods). *P. putida* MBD1 produced no detectable antibacterial or antifungal compounds under conditions where a positive control (*P. fluorescens* ATCC 49323) produced mupiromicin. Thus, *P. putida* MBD1 appears to provide a "clean" background for production and detection of antifungal and antibacterial compounds.

***E. coli-Streptomyces* shuttle BAC vectors can be transferred into and maintained in *P. putida* MBD1.** The RK2 system (used to introduce pMBD14 and derivatives into *Streptomyces*) is routinely used to transfer plasmids from *E. coli* to pseudomonads via conjugation. Therefore, we used standard protocols (see Materials and Methods) to conjugate DH10B/pUB307 containing pMBD14 with *P. putida* MBD1. Conjugation resulted in stable Apra-resistant *P. putida* MBD1 colonies, indicating that the ϕC31 integrase gene and the Apra resistance gene in pMBD14 were expressed and functional in *P. putida*.

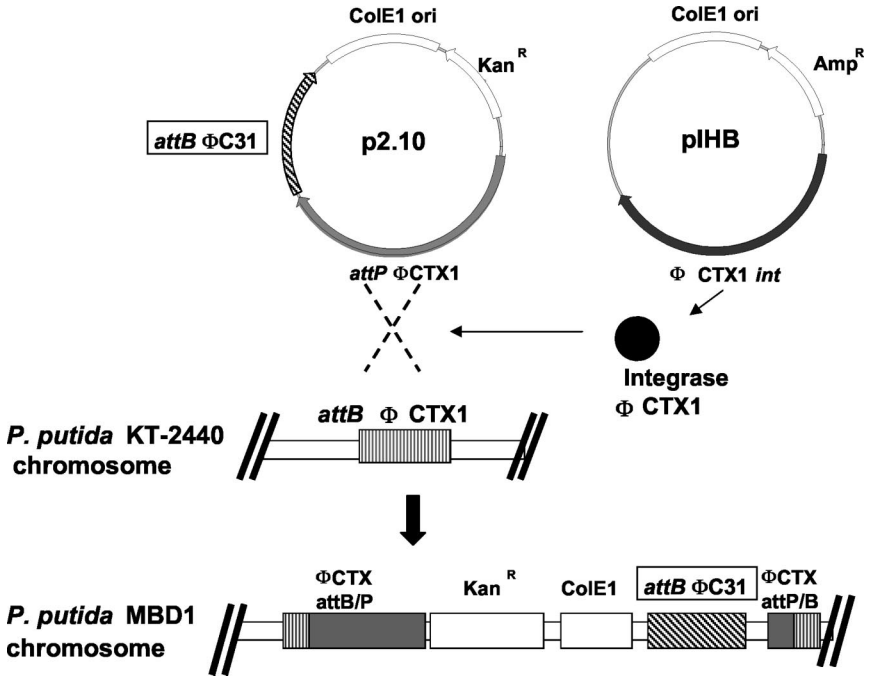

FIG. 4. Construction of *P. putida* strain MBD1. Plasmid p2.10, which contains the ϕC31 *attB* site and the ϕCTX *attP* site, and plasmid pIHB were cotransformed into *P. putida* KT-2440. Both are suicide plasmids, but the ϕCTX integrase encoded in pIHB can mediate recombination in *trans* between the ϕCTX *attP* and *attB* sites, resulting in the integration of p2.10 (and thus the *Streptomyces* phage ϕC31 *attB* site) into the chromosome of *P. putida* KT-2440.

To test the strain further, we conjugated shuttle BAC vectors containing DNA inserts of various sizes, including pSGran (37 kb), pSMG1.1 (27 kb), and pSDAPG (described below; 6.5 kb), as well as various plasmids from a soil DNA library constructed in BAC vector pMBD14, into *P. putida*. All of these vectors transferred easily into *P. putida* MBD1, regardless of the size of their insert DNA. Exconjugants containing one of the soil DNA clones, BamBAC8 (38-kb insert), were analyzed by Southern hybridization to demonstrate that the BAC vector integrated into the *P. putida* MBD1 chromosome at the ϕC31 site. Results (Fig. 5A) showed that the band containing the ϕC31 *attB* site in MBD1 (lane 2) was replaced in the exconjugants (lanes 3 to 5) by two new bands containing the *attL* and *attR* sites created by integration of BamBAC8 into the chromosome. The same two new bands also hybridized to the BamBAC8 probe, showing that they indeed contained the chromosome-integrated vector junctions. Other than the new junction bands, the band pattern following hybridization to the BamBAC8 probe in the exconjugants was identical to that of purified BamBAC8, demonstrating that no major deletions or rearrangements of the plasmid occurred in the 38-kb insert. The same results were obtained for *S. lividans* Δ*act*Δ*red* exconjugants (Fig. 5B).

These results confirmed that the *E. coli-Streptomyces* shuttle BAC vectors and large-insert environmental library clones can be introduced and maintained in *P. putida* MBD1, and they also provided the first example of BAC vectors that can be shuttled by conjugation from *E. coli* to both *Streptomyces* and *Pseudomonas*.

Heterologous expression of gene clusters in *P. putida* MBD1 versus that in *E. coli* DH10B and *S. lividans* Δ*act*Δ*red*. To explore the expression of heterologous gene clusters in the various host strains, we introduced into the three hosts a series of BAC constructs (pSgran, pSMG1.1, and pSDAPG) containing gene clusters encoding the synthesis of known antibiotics. *P. putida* MBD1 exconjugants containing these plasmids or the pMBD14 vector alone were grown for 6 days at 27°C, and ethyl acetate extracts were prepared and analyzed as described in Materials and Methods. DAPG was clearly detectable in the extracts of the *P. putida* MBD1 clone containing the pSDAPG construct (Fig. 6A). However, DAPG could not be detected in extracts of *E. coli* DH10B or *S. lividans* Δ*act*Δ*red* cells containing the same construct. Conversely, the products of the MG1.1 and granaticin gene clusters, expressed in *E. coli* and *S. lividans*, respectively, could not be detected in *P. putida* by either HPLC analysis of extracts or by antibacterial or antifungal assays. These results (summarized in Table 2) clearly underscore the advantages of the three-way conjugative shuttle BAC vectors and the use of multiple host systems in that the same genes can be transferred to a diverse set of bacterial hosts, thus increasing the chances of detecting expression of molecules of interest.

High-throughput transfer of environmental DNA libraries into *S. lividans* and *P. putida* and analysis of extracts. Our high-throughput method for transferring pMBD14-based large-insert libraries from *E. coli* to *S. lividans* (see Material and Methods) (Fig. 7) resulted in a 95% success rate for conjugation. We also developed a high-throughput conjugation method for *P. putida* MBD1 that can be performed in parallel

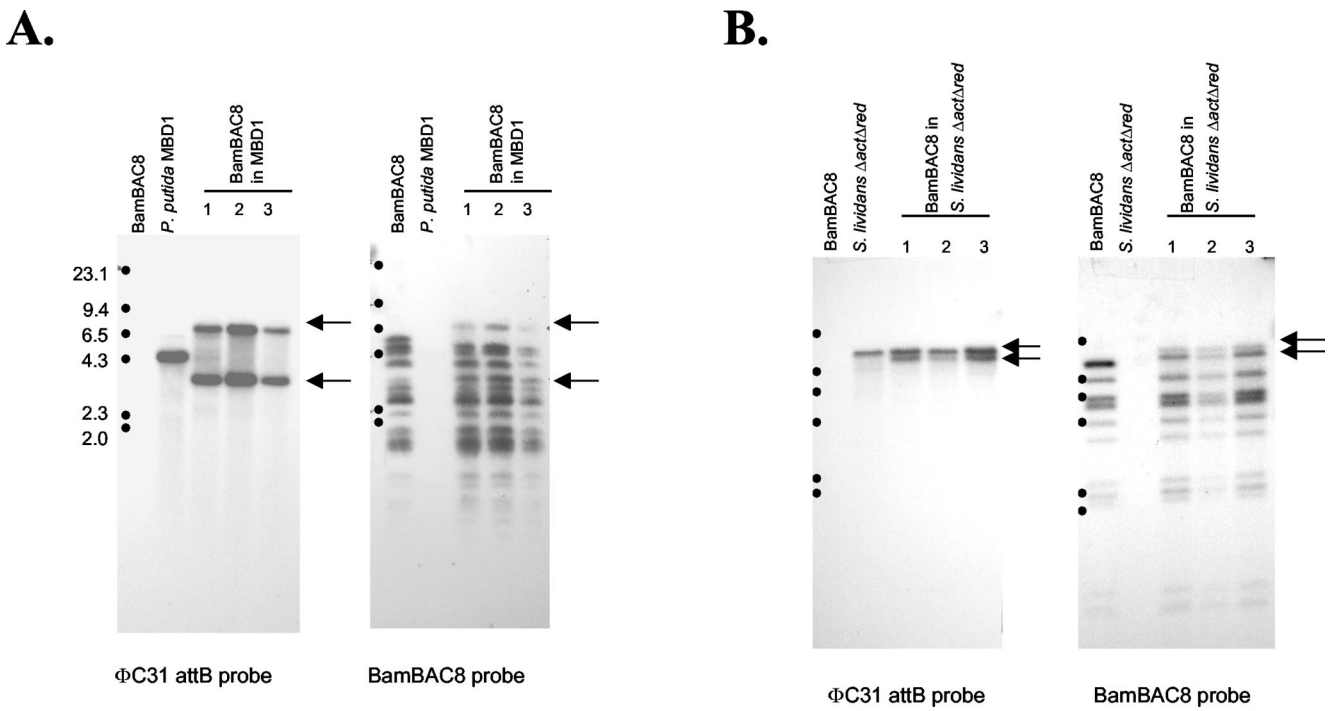

FIG. 5. Southern analysis of BamBAC8 exconjugants. (A) Chromosomal DNA of *P. putida* MBD1 and three BamBAC8 exconjugants was digested with HindIII and hybridized to the ϕC31 probe (left) or BamBAC8 probe (right). The first lane contains HindIII-digested BamBAC8 plasmid. (B) Chromosomal DNA of *S. lividans* Δ*act*Δ*red* and three BamBAC8 exconjugants was digested with NotI and hybridized to the ϕC31 probe (left) or BamBAC8 probe (right). The first lane contains NotI-digested BamBAC8 plasmid. In both cases, the band containing the ϕC31 *attB* site in the parental strain (lane 2) was replaced in the exconjugants by two new bands (marked by arrows) containing the *attL* and *attR* sites created by integration of BamBAC8 in the chromosome.

to the *E. coli-Streptomyces* conjugations (see Materials and Methods) (Fig. 7), with a success rate for conjugation of over 90%. The simplicity and efficiency of this transfer method thus enable the straightforward analysis of environmental libraries in all three hosts.

DISCUSSION

Accessing biodiversity by means of shotgun cloning environmental DNA is an exciting new technology that holds promise for natural products drug discovery. To facilitate this process, we expanded on our previous technology by devising new strains and vectors that enhance the chances of detecting activities encoded by environmental DNA.

In the course of this work, we developed new tools that are useful in both this and other applications. Our gene replacement vector contains a counterselectable marker for *Streptomyces* (*rpsL*) that allows positive selection of rare genetic events that lead to loss of plasmid sequences. This is an improvement over a previous *Streptomyces* gene replacement

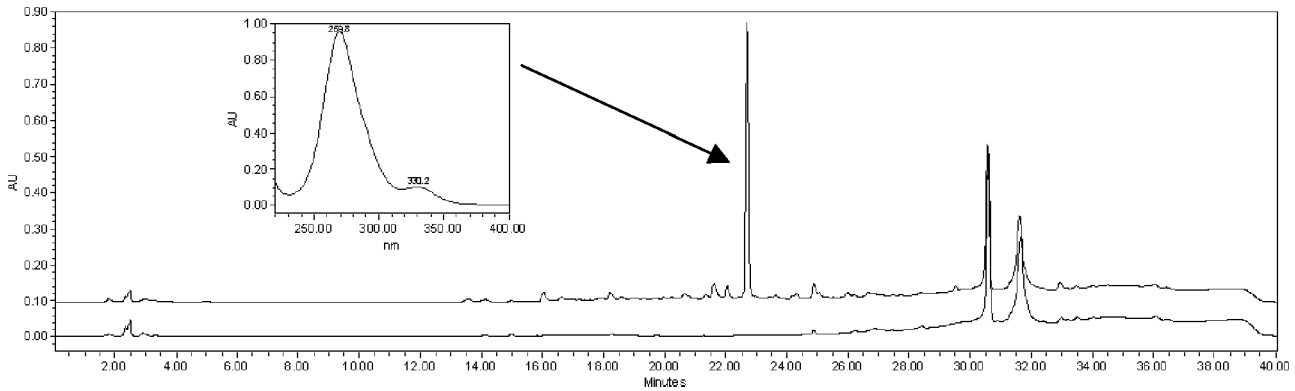

FIG. 6. Production of heterologous DAPG in *P. putida* MBD1, as shown by the reverse-phase HPLC elution profile at 270 nm for extracts of *P. putida* MBD1 exconjugants containing pMBD14 (lower trace) and pSDAPG (upper trace). The UV spectrum for the peak at a retention time of 22.7 min is shown.

TABLE 2. Comparison of heterologous antibiotic expression patterns for *E. coli* DH10B, *S. lividans* Δ*act*Δ*red*, and *P. putida* MBD1

Product	*E. coli* DH10B[d]	*S. lividans* Δ*act*Δ*red*[d]	*P. putida* MBD1[d]
MG1.1[a]	+	−	−
Granaticin[b]	−	+	−
DAPG[c]	−	−	+

[a] Production of MG1.1 was assessed visually by the presence of blue pigment and antibacterial activity in colonies.
[b] Granaticin production was assessed by the presence of purple pigment and HPLC.
[c] Production of DAPG was assessed by HPLC.
[d] The amount of extract analyzed corresponded to culture volumes of 0.5 ml (for *E. coli* and *P. putida*) and 1.2 ml (for *S. lividans*). See text for details.

plasmid, pRHB514 (14), because the new vector does not leave a drug resistance marker in the chromosome. This attribute is critical for many applications, including defining structure-function relationships and the production of vaccine candidates. In addition, the new vector can be used in successive rounds of gene replacement in the same strain without the need to use multiple drug resistance markers. Finally, the presence of the selection marker in the plasmid allows the excised molecule to be recovered, thus permitting the replaced allele to be isolated.

We have constructed *S. lividans* and *P. putida* host strains that are optimized to express environmental libraries. Our *S. lividans* strains contain complete and unmarked deletions of one or both pigmented antibiotic gene clusters (*act* and *red*), providing a cleaner background for heterologous expression, and no residual antibiotic resistance markers. Prior published *Streptomyces* strains (*S. coelicolor* CH999 [Δ*act::ermE redE60*] [20]) and K4-114 and K4-115 (*S. lividans* TK24 [Δ*act::ermE*] [39]) do not have deletions of both clusters. Our *P. putida* MBD1 host strain uses the ϕC31 integration system to integrate BAC vectors into the chromosome. We anticipate that this system can be extended to other host strains in the future, which will augment the panel of expression hosts available.

We developed BAC vectors that can be used to construct libraries containing large DNA inserts in *E. coli* and that can then be transferred by high-throughput conjugation to both *S. lividans* and *P. putida* MBD1. Importantly, we have shown that an environmental library generated in pMBD14 can be efficiently transferred to *S. lividans* and *P. putida* by conjugation, including clones containing inserts of up to 85 kb. To our knowledge, this is the first example of conjugative transfer of such high-molecular-weight plasmids from *E. coli* to *Streptomyces*. Sosio et al. (32) have constructed *E. coli-Streptomyces* shuttle BACs that also use ϕC31-mediated site-specific recombination to integrate in the *Streptomyces* chromosome, and they showed that inserts up to 120 kb can be introduced and maintained in *S. lividans*. Their vectors, however, do not contain the *oriT* sequence and thus have to be transferred into *Streptomyces* by protoplast transformation, which is not amenable to high throughput. The simplicity and high efficiency of the conjugative transfer method described here makes feasible the transfer and screening of entire large-insert DNA libraries in *Streptomyces* and *Pseudomonas*. Environmental DNA clones can be transferred on a one-to-one basis using this process, enabling the *E. coli* counterpart of any interesting *Streptomyces* or *Pseudomonas* clone to be easily identified.

Environmental libraries offer a potentially rich source of novel and useful natural products. However, converting this intriguing idea into a realistic discovery program is a challeng-

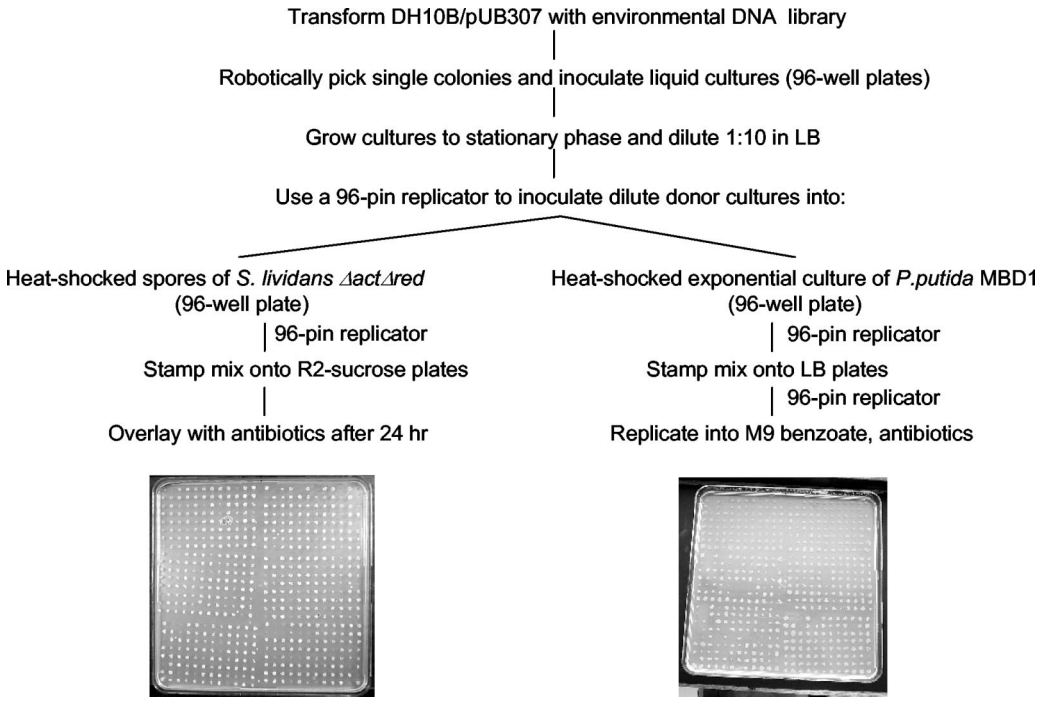

FIG. 7. High-throughput conjugative transfer of BAC libraries into *S. lividans* Δ*act*Δ*red* and *P. putida* MBD1. Plates exhibiting typical conjugation efficiencies are shown. See text for details.

ing endeavor. Prior data concerning the frequency of genes and gene clusters in environmental libraries of various sizes have been published by us and others (9, 17, 26). For example, one *E. coli* BAC library generated a hit rate for antibacterial activities of roughly 1 antibacterial clone per 60 Mb of soil-derived DNA (17). Another library of 5,000 cosmid clones yielded 11 partial clusters with homologies to type I polyketide synthases (9). Based on these frequencies, it is our view that in order to maximize the chances of discovery, environmental libraries need to be generated continuously and screened in a high-throughput fashion, using as many different expression hosts as practical. The strains, vectors, and technologies reported here provide an important step forward by offering practical solutions to increasing both the host range and the throughput for screening environmental libraries. The data presented here demonstrate that the three expression hosts (*E. coli*, *S. lividans*, and *P. putida*) differ in their abilities to express gene clusters encoding chemically diverse small molecules and should, thus, facilitate the capture of increasingly numerous and diverse natural product activities, greatly increasing the chances of success for this innovative technology.

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